BIO-REMEDIATION OF MONOCROTOPHOS PESTICIDES BY ACTINOBACTERIA

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1. Introduction and Background

Agriculture contributes immensely to Indian Growth Development Product (GDP) and a large population gets employment in agriculture sector in India which is also responsible for the growth and enhancing standard of life of millions. Majority of industries for raw materials depend upon the agriculture, for example sugar industry, fruits and vegetable processing industry, cereals, legumes and oil seeds processing industry, beverage industry, cottage industry etc. India being an agricultural country the utilization of pesticides has enormously increased in our agricultural activities. Pesticides are basically chemical complexes that are responsible for the elimination of pests. Pests could be bacteria, fungi, worms, nematodes or insects that are responsible for the deteriorating of the crops existing in the farms and also for declining quality of soil texture. Therefore, pesticides are widely used to control or prevent plant diseases and pests. Pesticides are essential for promotion of crop productivity but the surplus amount of chemicals leads to ecological contamination, bacterial imbalance and health issues. Therefore, a typical pesticide should be capable of bio-degradable quickly as possible and their end products must be non-toxic[1].

1.1 Role of pesticides in agriculture

In India, Pesticides are crucial for production in agriculture. About 22% of our country's GDP is contributed by the agricultural sector and is responsible for fulfilment of 70% nation's livelihood. Today in a global scenario some of the top five agrochemical companies in the world have captured the 60% of the market

share. As per a study conducted in 2006 there were large quantity of pesticide used in agriculture production which accounted for around 67%of the total consumption [2].Preferably a pesticide must have a fatal impact on the target pests, but it should not be lethal for non-target species, specifically humans. But unfortunately, due to inefficient management of pesticides, results in occupational health hazards and environmental problem posing risks for different ecosystems [3].Further, pesticide residues remain in soil which are taken up by the crops can have direct impact on human health via consumption of food containing pesticidal residues. A report by The World Health Organization suggested million cases of pesticidal poisoning worldwide, provides sufficient data that pesticide effect different domains of human health pathologically[2]. Further rise in pesticidal pollution is resulting in the emergence of 'cancer villages'. The term cancer village denotes to a region where death rate due to cancer is significantly greater than the average mortality rate, mainly because of widespread contamination of pesticide, chiefly in water[4].On the basis of their toxicity, the most toxic pesticides are organophosphorus (OP) followed by carbamate (CB), and organochlorine (OC) pesticides. These are the predominant complex chemicals that initially act by causing nervous system disorders [5].The man-made organic compound has created so much pollution in the environment therefore there is an urgent need to come out with immediate solutions to save our environment. There is a list of around 104 pesticides which have been restricted for use in other parts of the world but in India these pesticides are still being used which causes a serious concern for people. However the Government of India is considering to review only 66 of these pesticides. The Ministry of Agriculture, Govt. of India on 8 August, 2018 put out a gazette notification banning 18 neonicotinoid pesticides in

the country. Around, 12 pesticides have been banned with immediate effect; six will be phased out by December 2020[6].

1.2 Impact of Monocrotophos pesticides on human health

There are various harmful pesticides available and vastly in India for agriculture purpose and monocrotophos is one of them which is easily available in the market for use. This pesticide is widely used in crops like castor, olives, rice, maize, citrus, sorghum, soybeans, sugar beet, sugar cane, peanuts, potatoes, onion, beverages, spices, and tobacco. The excessive use of such pesticides causes the maximum soil stress and infertile conditions. The accumulation of such pesticides also imbalances the water level in the water table and the natural degradation of the same is not an easy task. Only bioremediation is a perfect solution which takes 12-16 days duration for bio-degradation [7]. As per WHO (2009) report monocrotophos pesticides are the major reasons for the accidental poisonings. The state wise report on monocrotophos pesticides published in 2001-2006 revealed highest usage by Andhra Pradesh (2,779 metric tons) followed by Punjab (1,274 metric tons), Gujrat (865 metric tons), Haryana (823 metric tons), Karnataka (624 metric tons), Madhya Pradesh (597 metric tons), Tamil Nadu (522 metric tons), Rajasthan (512 metric tons), West Bengal (169 metric tons), Kerala and Bihar (103 mega tons).

609 deaths were reported due to the consumption of organophosphorus pesticides, 86 due to intake of monocrotophos in India out of total 1531 deaths that were

reported in India in the year 2000. Of these it was found that the monocrotophos was highly poisonous and was also responsible for maximum number of casualties.

Monocrotophos is poisonous organophosphates observed all cross the country and are widely used for agriculture. It can be penetrated thru the skin which is also a direct acting cholinesterase catalyst. Though, it's Symptoms are same as those of other Organophosphate compounds but it may take few minutes or even a day to notice the effect. It is further stated that the Cholinesterase constraining action affects the nervous system. There are various symptoms of poisoning such as muscular weakness, profuse perspiration, blurred vision, confusion and vomiting etc. This may involve vomiting, diarrhoea, nausea, headache, abdominal cramps etc. Severe poisoning due to monocrotophos causes cardiac arrest or respiratory failure which leads to death of person in the severe cases [2]. The two main Organizations related to health and agriculture (FAO) and WHO encouraged countries to list out pesticides having highly hazardous components. The use of monocrotophos has been forbidden in many nations like USA, china European union, Thailand. Vietnam, Srilanka, Australia, Philippines, and Indonesia. To take off this from market urgent steps should be taken. Not only the developed countries in Europe and America but also, many countries in Asia have also barred the use monocrotophos due its highly devastating and ill effect on human health. India is very much familiar with the threats of pesticides. But in the fields of rural India, pesticides like monocrotophos is continuously produced, used and exported in India. The reason behind this is that it is cheap and necessary for agricultural productivity [8]**.** The image of chemical structure of monocrotophos pesticide is shown in **Image 1.**

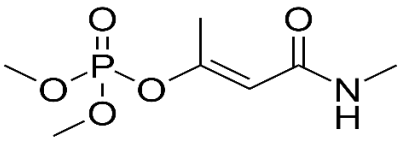

Image 1: Chemical structure of Monocrotophos ($C_7H_{14}NO_5P$)

1.3 Bioremediation: the most eco-friendly Approach for the degradation of xenobiotics

For cleaning up the contaminated environment different eco-friendly methods have been used in last two decades, such as use of different microbial species. This method is known as bioremediation. As compared to conservative physicochemical methods, this is considered as more desirable and more restorative of soil functions[9].Bioremediation is also used to convert the hazardous substances into less toxic components. Microorganisms have a vital role in the breakdown and mineralization of these contaminants. Bioremediation as sustainable technology is significant in examining and reducing manmade chemicals released into the environment. Bioremediators are the microorganisms used to attain the function of bioremediation. Bioremediation technology has been commonly categorised as *ex situ* and *in situ* bioremediation. In situ bioremediation comprises the treatment and elimination of the contaminated material at the site, whereas in ex situ the contaminated are treated somewhere else. Bioventing, bioleaching, biostimulation,

land farming, composting, bioaugmentation, rhizofilteration and phytoremediation are few examples of bioremediation technologies [10].

Biodegradation and bioremediation are similar methods upto an extent since both of these approaches employs microorganisms for the alteration or breakdown of pesticide. The only difference between these two is that the biodegradation is served as a natural process whereas bioremediation is considered as technology. There are various aspects which turns out to be limiting factors for pesticide degrading microorganism such as pH, temperature, nutrients, water potential and amount of metabolites or pesticide content in soil [11].

1.4 Actinobacteria: the most promising candidate which must be overlooked for bioremediation

Bioremediation of organic compounds and heavy metals has been recognized to be successful, although at the moment each process has normally been performed separately. For the bioremediation of co-contaminated spots, broadly a thought process has been going on among experts that a multifunctional biological process may be required and it needs further research. After having a closer look it can be further noticed that the bacteria of the phylum Actinobacteria reproduce great potential considering the fact that they have been found suitable not only bioremediation of pesticides but it can further help in case of heavy metals. Presently, it's been noticed that various studies are being made on the constituents of the phylum Actinobacteria because of their potent ability to bioremediate the soils that are co-polluted [12]. It is established that, Actinobacteria is the diversified phylum having major 6 classes, 19 orders, 50 families and 221 genera. The

actinobacteria are Gram positive, high G+C rich bacteria [13]. The member of the phyla, *Streptomyces sp* is a rich source of natural products which is able to secrete enzymes and secondary metabolites which behaves as antimicrobial agents and anti-tumour agents. These well-known organisms, Actinomycetes are the source of 45% of all bioactive secondary metabolites and 80% of the composition is produced only by *Streptomyces* genera[14].These also produces non antibiotic molecules which exhibits bioactivity such as immuno-suppressors, Phyto-toxins, bio-pesticides, Nano-particles, probiotics, enzyme inhibitors and different enzymes and proteins which are involved in degradation of complex polymers and biomolecules [15]. Although few of the xenobiotic pesticides that have been discussed here are made to be mineralized by single isolates, but it can be estimated that many of this particular group of bacteria are actually required to perform a complete degradation process. Having a closer view of this group of bacteria, it is found that the co metabolism of pesticides established in most of the experiments. On the other hand when we take another example by trying different experiment which involves molecular mechanisms in biotransformation of pesticides by actinomycetes and relate it with pesticide degradation then Gr -negative bacteria is produced. In the absence of a suitable molecular genetic tools a vast number such agents of this major group of soil bacteria's growth has been immensely hindered. We further notice that a much better utilization of the biodegradation and biotransformation experiences of actinomycetes has not been improved. Therefore, a suitable application looks far from reality for bioremediation and creation of transgenic herbicide-resistant crops, although it's very much needed for further development and growth at present [17]. Actinobacteria has immense biotechnological potential as it is capable of eradicating

organic as well as inorganic pollutants. Due to which it has gained much attention in recent years, thus are preferred ideally for bioremediation [18].

1.5 Review of studies done on biodegradation of pesticides

A report on *Arthrobacter atrocyaneus* MCM B-425 and *Bacillus megaterium* MCM B-423, states that the isolates in question are capable of degrading MCP to carbon dioxide, ammonia and phosphates to the range of 93%and 83% correspondingly [19]. With respect to genera Actinobacteria, which are cosmopolitan found both at aquatic as well as terrestrial habitat. Previous studies clearly indicate that it plays a vital ecological role in recycling and degradation of bioactive molecules; it has also potential to remove organic as well as inorganic pollutants from the environment, due to its unique properties research workers get attracted toward it and pay attention in the field of bioremediation, for the reason that environmental pollutants are widespread through worldwide.

Pesticides widely used for pest control throughout the world and due to this it has aroused as main organic contaminants which spread globally and due to excessive uses of it showed negative impact on environment which is increasing through the time. With respect to heavy metals, extensive use of it in industries leads to major contaminants globally. Due to hazardous effect on the human population of these environmental pollutants workers focused on those problem and searched the solution for it, since last 15 years workers have focused on developing the tools for cleaning the environment. On the basis of previous research reports it is clear that actinobacteria has potential to bioremediation. Researchers tried different ways to enhance and utilize the bioremediation potential of actinobacteria through cell

immobilization biostimulation, bioaugmentation, production of biosurfactants, design of defined mixed culture and the use of plant-microbe systems, etc [20] . As it is well significant that Actinomycetes have capability for biodegradation and biotransformation of pesticides. Gram positive bacteria of this group have been found to effective against different chemical structures

(s-

triazines, triazinones,carbamates, organophosphates, organophosphonates, acetanilid es, and sulfonylureas). However, bioremediation of xenobiotics pesticides from single bacterial isolates is limited rate, so different bacterial consortia is required to complete degradation of pesticides.

The Metabolism pertaining to pesticides is recurrently seen in the group of bacteria. Though we do not find enough information about molecular mechanisms that is involved in the process of bio-transformations of pesticides by actinomycetes. This is clearly indicated when compared with pesticide degradation by gram-negative bacteria. This has been further noticed that there is a shortage of proper molecular genetics tools for most of constituents of this group of soil bacteria. This phenomena which is continuing for a long time. There is a need of further research and a most suitable solution is desired to fully exploit the biodegradation and biotransformation abilities of actinomycetes for bioremediation and construction of transgenic herbicide-resistant crops [21]. This is particularly needed in a country like India where still around 55% of population is dependent on agriculture for their livelihood.

As these microbes work as slow rate and has less bioremediation potential in their natural environment. To increase the efficiency of these microbes some modifications is required so that it degrade the pesticide at a faster rate in a limited time period, so that we used these microbes and utilize it for removal of contaminant from its origin. As we know that specific gene encodes specific enzyme which are identified by specific pesticides this ability provide the information to understand microbial potential to degrade a pesticide and develop a strain to achieve the enhance bioremediation process of desired microbes with precision [22]. There are number of studies that have explained the effects of genotoxic and cytotoxic monocrotophos which is an organophosphate insecticide found on cultured human lymphocytes. These studies proved that monocrotophos are considered to be exceedingly toxic to lymphocyte culture. This is further indicated that the chromosomal aberrations that were prompted by monocrotophos were the result of using karyotyping. When we study is further and compare to controls, it is found that more satellite associations, breaks and gaps were noticed and these were significant when we assess and analyze it empirically (P 0.05) [22].

At global level, approximately 34% of organophosphorus compounds are to be used accounting from insecticidal sale. We also noticed during these studies that in ground water or soil, a pesticide contamination is generally found because a large quantity of pesticides has been used in the fields during agriculture activity or that may have been slipped in the fields accidently. Deeper study also suggests that organophosphorus contain toxicity which is extremely harmful for human body and it has been noticed that different water bodies that are existing may have been contaminated due to its excess use which is not only limited to this but also the

whole ecosystem may be in danger due to its presence which needs to be arrested with immediate effect to save the whole ecosystem.

Therefore, bioremediation may offer a useful tools for decontamination of these toxic pollutants, because bioremediation is reasonably affordable for the public in general. In 1973, first isolate of the microbes which successfully degrade the organophosphorus later recognized to be *Flavobacterium sp.* The study clarifies that very few bacterial and fungal species may have been isolated which gives a greater scope and space for reduction of or full degradation of organophosphorus in the water or soil values [23] .Out of tis most of the bacteria are found to be having an enzyme organophosphate hydrolase or phosphotriesterase which leads to the clarification on the first step of the organophosphorus compound deprivation. It is further noticed that this enzyme encode gene opd (organophosphate degrading) has been inaccessible by the species found in the area of experiments. This particular gene is found to be cloned in various other organisms and has further been modified for a more suitable activity and sustainability. We also found that in most recent times scientists and a number of researchers have been able to isolate the genes with parallel function but the sequences have been found to be slightly different [24]. Different researches reported the bioremediation of monocrotophos pesticide (MCP). The isolates were obtained after having growth on 1% agar medium containing monocrotophos pesticide. These isolates were found to have MCP degradation capabilities and are found to be bacterial and fungal strains respectively[25]. A study further suggested where the isolates were collected from the sludge available in the wastewater treatment system of a chemical factory and a research was conducted on the cellular morphology, physiological and chemotaxonomic which identified a

stain to be a *Paracoccus sp*. These are some of the suggestive studies but further scope of research is available on this subject.

It has potential to mineralize monocrotophos in culture conditions. It has also ability to degrade the other organophosphorus insecticides and amide herbicides, it reported that M-1 are the key enzyme which involved in the biodegradation of monocrotophos and expressed as cytosolic protein [26].

2. Hypothesis

Monocrotophos are still to be restricted in India, although this is banned for use by world health organization, because it's cheaper than the other available pesticides in the market and also, it's effectiveness has been better than the co-existing pesticides in the market place. Major population of the states of India does not have the access to quality crops even after the huge investments and massive efforts, with respect to higher levels of monocrotophos contamination in rural as well as urban areas. The higher indirect consumption of this pesticide results in deaths in India. The pesticides that are being sold in India are directly acting as a cholinesterase which is a catalyst quite effective in penetration through the skin. Though, it's Symptoms are same as those of other Organophosphate compounds but it may take few minutes or even a day to notice the effect. It is further stated that the Cholinesterase constraining action affects the nervous system. Severe poisoning due to monocrotophos causes cardiac arrest or respiratory failure which leads to death of person in the severe cases. However, till now no effective methods are available for the bio-degradation of monocrotophos pesticides, as they persists in the soil and water for longer durations and their contact with humans and animals causes severe diseases and deaths.

Therefore, there is an urgent need for the bio-degradation of such type of pesticides in to nontoxic forms. In this scenario, the application of actinobacteria in bioremediation of such types of pesticides may be an effective method because the metabolic diversity of such type bacteria is huge and exploiting their metabolic diversity may prove to be an important step in bio-degradation of monocrotophos in to non-toxic forms. The actinobacteria isolates may be useful in biodegradation of monocrotophos pesticides and product based on such microbial consortia may be utilized for bioremediation of chemicals influenced soil. The Bio-formulation of the promising candidates (if found) of this study may patented.

3. Objectives of the study

The genera of microbes which include, *Streptosporangium*, *Nocardiodes*, *Actinomadura*, *Planobispora*, *Micromonospra*, *Saccharopolyspora* etc are rarely exploited in bioremediation of pesticides. In this context, the present study have been designed to isolate the rare genera of actinobacteria degrading the monocrotophos pesticides in to nontoxic forms and their bio-formulation and application in agriculture with following objectives:

1. Isolation and screening of actinomycetes for bioremediation of Monocrotophos pesticides (MCP) as well as for their PGPR traits.
2. Identification of promising isolates using polyphasic taxonomy
3. Characterization of degraded products of Monocrotophos pesticides and to assess their toxicity

4. Expression of genes in *E.coli* system and their application in bioremediation (*In vivo*)

4. Methodology

(a) Collection of soil samples

(b) Selective and non-selective isolation of rare genera of actinobacteria.

  (a) Selective: Soil pretreatment methods

    Use of antibiotics

    Use of selective media

  (b) Non- selective isolation

(c) Screening for their ability to degrade the monocrotophos pesticides and for their PGPR traits.

    (a) Primary Screening

    (b) Secondary Screening[27]

(d) Characterization of most promising candidates by polyphasic taxonomy

    (a) Cultural characteristics

    (b) Morphological

    (c) Physiological

    (d) Molecular-16S rDNA analysis, housekeeping genes (MLSA/MLST)[28]

(e) Study of biodegraded products by GC-MS, HPLC and to assess their toxicity[29]

(f) Bio-formulation with suitable carriers and to check their viability at several durations.

(g) Filed trials of bi- formulations to assess their efficacy.

(h) Statistical analysis.

5. Proposed Outcomes

(i) The Bio-formulation of the present study may be used for the remediation of Monocrotophos pesticides as well as for increasing the agriculture yield, which may be an eco-friendly approach based on actinobacteria. As per the literature survey, very few or no products have been patented or registered so for based on the proposed study.

(ii) Product may be registered or patented.

(iii) The present study will explore different microbial isolates for soil detoxification and bioremediation against the monocrotophos pesticide.

(iv) The present study will arise new aspect of bioremediation in terms of biological agents that can be utilized to degrade such chemical toxicity in soil.

(v) The present study will generate new avenues and prospects in exploring the versatile microbial diversity in soil samples of different geographical location for defining the possibility in biodegradation of such chemical pesticides.

(vi) The study will prove to be a basis in exploring novel microbes(PGPRs) and their metabolites that can be found worth as the growth promoting, fungicidal and pesticidal agent (s).

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